United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

SAM 901

Supplemental Assay Method for Testing Growth-Promoting Qualities of Fluid Thioglycollate Medium with Beef Extract Using *Clostridium chauvoei* Spores as the Indicator Organism

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Supplemental Assay Method for Testing Growth-Promoting Qualities of Fluid Thioglycollate Medium with Beef Extract Using *Clostridium chauvoei* Spores as the Indicator Organism

1. Introduction

This is a Supplemental Assay Method (SAM) for testing Fluid Thioglycollate Medium with Beef Extract for growth-promoting qualities as required in the Code of Federal Regulations, Title 9 (9 CFR), Part 113.25(b). Each lot of media that is used in sterility tests (9 CFR 113.26 – 113.27) must be tested to ensure that it will support the growth of contaminants, should they be present in the biologics sample being tested for sterility. Fluid Thioglycollate Medium with Beef Extract is one of the media used in codified sterility tests.

2. Materials

2.1 Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

2.1.1 30º- 35ºC incubator

2.1.2 Sterile disposable cotton-plugged pipettes

2.1.3 Sterile 10-mL disposable syringes, with needles

2.1.4 Sterile glass tubes, 25 x 200-mm, with sterile closures

2.1.5 Class II biosafety cabinet

2.1.6 Freezer, -70°C or lower

2.1.7 Magnetic stirrer

2.1.8 Anaerobic Growth Chamber

2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below.

2.2.1 Indicator organism: *Clostridium chauvoei* spores or equivalent organisms as specified in the current United States Pharmacopoeia (USP).

2.2.2 Media: Fluid Thioglycollate Medium with 0.5% Beef Extract (FTM/BE) Soybean Casein Digest Medium (SCDM), and Beef Infusion Agar Medium. See the **Appendices** for media formulations.

2.2.3 Sterile 0.85% NaCl (**Appendix III**)

2.2.4 0.015M Phosphate Buffered Saline, pH 6.9 (**Appendix V**)

3. Preparation for the Test

3.1 Personnel qualifications/training

Personnel must have experience or training in this protocol. This includes knowledge of aseptic biological laboratory techniques and preparation, proper handling, and disposal of biological agents, reagents, tissue culture samples, and chemicals. Personnel must also have knowledge of safe operating procedures and policies, as well as training in the operation of the necessary laboratory equipment listed in **Section 2.1**.

3.2 Preparation of equipment/instrumentation

3.2.1 Turn biosafety cabinets on at least 30 minutes before preparing positive control reagents or testing media for growth promotion.

3.2.2 Monitor incubators, freezers, and coolers used for the storage of reagents and controls for temperature daily.

3.3 Preparation of *Clostridium chauvoei* reagent

3.3.1 Cultivate *C. chauvoei* spores on the surface of beef infusion agar medium (**Appendix IV**) in a 500-mL flask. The flask is incubated in an anaerobic chamber containing 85% nitrogen, 10% hydrogen, and 5% carbon dioxide at 35°C for 2 to 4 days.

3.3.2 Transfer the flask to 25°- 28°C for 4 days.

3.3.3 Harvest the spores by washing the agar surface with sterile 0.015M phosphate buffered saline, pH 6.9 (**Appendix V**). Mix the spore suspension with an equal volume of sterile glycerol and dispense into tubes for storage at -70°C or colder. No expiration date is assigned to this product because the spores have demonstrated over time that they will retain their viability.

3.3.4 Place 1 mL of *C. chauvoei* spore suspension from **Section 3.3.3** stock culture into 100 mL of 0.85% saline (**Appendix III**) and stir on a magnetic stirrer for 30 minutes at room temperature.

3.3.5 Dispense 1.5-mL aliquots of this diluted stock culture (spore suspension) into sterile screw-cap tubes. Label the tubes with the reagent name, lot number, and date. Store frozen at -70ºC or colder. This is referred to as a diluted stock culture in the remainder of the document. Label and fill records are filed according to standard operating procedures.

4. Performance of the Test

4.1 Establishing the working dilutions of the indicator organism

Titrate each new lot of diluted stock culture of *C. chauovei* to determine the optimum working dilution. Once established, this working dilution will then be used to test unknown lots of media for growth-promoting qualities.

4.1.1 Remove a vial of the newly prepared diluted *C. chauvoei* stock culture from the freezer and thaw rapidly.

4.1.2 Make tenfold dilutions (1 mL into 9 mL) of the diluted stock culture in FTM/BE until a 10^{-10} dilution is prepared.

4.1.3 Incubate the *C. chauvoei* dilution tubes for 24 to 48 hours at 30º- 35ºC.

4.1.4 Examine the tubes visually for growth to establish the growth endpoint of the stock culture. The growth endpoint is the last tube where visible growth is observed.

4.1.5 The growth endpoint and the next lower dilution are used for testing the growth-promoting qualities of new lots or batches of media.

4.1.6 Collect data to confirm the reproducibility of the growth endpoint in the assay system (**Section 4.1.5**). Perform two independent tests with each of five valid (i.e., already satisfactorily tested for growth promotion) lots of media (total of 10 tests). Use a separate vial of stock culture for each test.

4.1.7 Growth is expected in 9 or 10 tubes inoculated with the lower dilution and in less than 9 tubes inoculated with the higher dilution.

4.1.8 If all of the tests give the expected number of tubes with growth, then these dilutions will be used to test new lots or batches of media for growth promotion. If these results are not repeatably achieved, adjust the dilutions and retest according to **Section 4.1.6.**

4.2 Testing new lots of media for growth promotion

4.2.1 Thaw a tube of diluted *C. chauvoei* stock culture rapidly.

4.2.2 Prepare tenfold dilutions (1 mL into 9 mL) of the diluted *C. chauvoei* stock culture in SCDM. Increase the transfer volume to 3 mL into 27 mL SCDM for the final two dilutions, so that a sufficient volume of each working dilution (**Section 4.1.5**) is prepared.

4.2.3 Inoculate 1.0 mL (using a sterile 10-mL pipette or syringe with needle) of the higher working dilution into each of 10 tubes (25 x 200-mm) containing 40 mL of FTM/BE. Inoculate a similar set of 10 tubes with the lower working dilution.

4.2.4 Incubate all tubes (20) at 30º- 35ºC and observe for growth of the organism throughout the 14-day incubation period.

4.2.5 Thoroughly clean the work area after completing the entire procedure.

5. Interpretation of the Test Results

If at least 9 or 10 of the tubes inoculated with the lower working dilution of a stock culture contain growth, the growth promoting quality of that medium is satisfactory (SAT). If less than 9 tubes at the lower working dilution have growth, then the growth promoting qualities of the media are in question and the test must be repeated. If after repeating the test and the media's growth promoting properties are still in question, the media must not be used and all tests already conducted with this media must be considered no tests (NT).

6. Report of Test Results

Report results of the test(s) as described by standard operating procedures.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.25, U.S. Government Printing Office, Washington, DC.

7.2 The U.S. Pharmacopoeia, 1985, Vol. 21, pp 1151-1160, Mack Publishing Co., Easton, PA.

8. Summary of Revisions

This document was revised to clarify practices currently in use at the Center for Veterinary Biologics and to provide additional detail. While no significant changes were made that impact the outcome of the test, the following changes were made to the document:

- The document number has been changed from STSAM0901 to SAM 901.
- The Contact has been changed from Gerald Christianson to Sophia G. Campbell.
- **1:** Information to clarify the testing purpose has been added.
- **2.1.4:** Sterile glass tubes have been added to the equipment list.
- **2.1.5**: The class of biosafety cabinet to be used has been added.
- **2.1.8**: An anaerobic growth chamber for use in *C. chauveoi* propagation and spore harvest has been added.
- **2.2**: The list of reagents/supplies has been updated.
- **3.1:** Personnel qualifications have been clarified.
- **3.3:** Information on the growth of *C. chauveoi* culture and harvest of spore suspension has been added.
- **4.1/4.2:** These sections have been revised to clarify the procedures followed in testing.
- **5:** The test interpretations have been clarified.
- **Appendices:** Media storage conditions have been added.
- **Appendix III:** A temperature range has been added for the storage of Normal Saline.
- **Appendix IV:** This section has been added to provide the media recipe for beef infusion agar medium.
- **Appendix V**: This section has been added to provide the media recipe for 0.015M phosphate buffered saline pH 6.9.

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Appendices - Media Formulations

Appendix I

Fluid Thioglycollate with Beef Extract--National Veterinary Services Laboratories (NVSL) Media #10227

Fluid Thioglycollate Medium	29.5 g
QH_2O	1000 mL

Heat and add:

0.5% Beef Extract (Difco)	5 g

Bring to a boil and dispense. Autoclave 20 minutes at 121ºC. Store at 20°- 25°C for no longer than 3 months.

Appendix II

Trypticase Soy Broth (TSB) or Soybean Casein Digest Medium (SCDM)--NVSL Media #10423

Trypticase Soy Broth	30 g
QH_2O	1000 mL

Autoclave 20 minutes at 121ºC. Store at 20°- 25°C for no longer than 3 months.

Appendix III

Saline, 0.85% (Normal Saline)--NVSL Media #30201

Sodium Chloride	8.5 g
QH_2O	1000 mL

Autoclave for 20 minutes at 121°C. Store at 2°- 5°C for no longer than 6 months.

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Appendix IV

Beef Infusion Agar Medium--NVSL Media #10317

Beef Infusion 2X Stock Solution #40001	500.0 mL
QH_2O	500.0 mL
Bacto Peptone	10.0 g
Sodium Chloride	5.0 g

Mix to solution, then adjust pH to 7.6. Autoclave 30 minutes at 121ºC. Filter through #2 Whatman paper. Check final pH and adjust if needed (should be 7.3).

Add:

Agar	15.0 g

Bring to a boil and dispense. Autoclave again for 20 minutes at 121ºC. Store at 2°- 5°C for no longer than 3 months.

Beef Infusion 2X Stock Solution -- NVSL Media #40001

Ground Chuck	1.0 lb
QH_2O	500.0 mL

On day 1, mix ingredients and let stand covered in refrigerator overnight. On day 2, heat mixed ingredients to a boil and reduce heat. Stir occassionally, but keep between 80° and 90ºC or slightly above for 1 hour. Bring to a boil again for 3 to 5 minutes, cover, and let stand for approximately 2 hours to settle meat. Drain through several thicknesses of gauze and then through coarse filter paper. Dispense into 4 liter jugs with sponge stoppers and paper covers. Autoclave 30 minutes at 121ºC. Some sediment may form during autoclaving, so filter through Whatman paper to remove before use. Use immediately or store at 2°- 5°C for no longer than 3 days.

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Appendix V

0.015M Phosphate buffered Saline, pH 6.9--NVSL Media #30178

Sodium Chloride	8.5 g
Potassium Chloride	0.2 g
Sodium Phosphate Dibasic	1.03 g
Potasium Phosphate Monobasic	0.2 g
QH_2O	1000 mL

Mix all ingredients. Bring up to 1000 mL, then pH to 6.9. Dispense as requested. Autoclave 20 minutes at 121ºC. Store at 2°- 5°C for no longer than 6 months.